Catalytic Acid−Base Groups in Yeast Pyruvate Decarboxylase. 2. Insights into the Specific Roles of D28 and E477 from the Rates and Stereospecificity of Formation of Carboligase Side Products†

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Received December 18, 2000; Revised Manuscript Received April 17, 2001

ABSTRACT: Yeast pyruvate decarboxylase (YPDC), in addition to forming its metabolic product acetaldehyde, can also carry out carboligase reactions in which the central enamine intermediate reacts with acetaldehyde or pyruvate (instead of the usual proton electrophile), resulting in the formation of acetoin and acetolactate, respectively (typically, 1% of the total reaction). Due to the common mechanism shared by the acetaldehyde-forming and carboligase reactions through decarboxylation, a detailed analysis of the rates and stereochemistry of the carboligase products formed by the E477Q, D28A, and D28N active center YPDC variants was undertaken. While substitution at either position led to an approximately 2−3 orders of magnitude lower catalytic efficiency in acetaldehyde formation, the rate of acetoin formation by the E477Q and D28N variants was higher than that by wild-type enzyme. Comparison of the steady-state data for acetaldehyde and acetoin formation revealed that the rate-limiting step for acetaldehyde formation by the D28A, H114F, H115F, and E477Q variants is a step post-decarboxylation. In contrast to the wild-type YPDC and the E477Q variant, the D28A and D28N variants could synthesize acetolactate as a major product. The lower overall rate of side-product formation by the D28A variant than wild-type enzyme attests to participation of D28 in steps leading up to and including decarboxylation. The results also provide insight into the state of ionization of the side chains examined. (*R*)-Acetoin is produced by the variants with greater enantiomeric excess than by wild-type YPDC. (*S*)-Acetolactate is the predominant enantiomer produced by the D28-substituted variants, the same configuration as produced by the related plant acetolactate synthase.

Yeast pyruvate decarboxylase isolated from the strain *Saccharomyces cerevisiae* (YPDC,[1] EC 4.1.1.1) is an enzyme consisting of 4 identical subunits of 563 amino acids each. It performs the prototypical decarboxylation of 2-oxo acids using thiamin diphosphate (ThDP, the vitamin B1 coenzyme) and Mg(II) as cofactors and converts pyruvate to acetaldehyde in the penultimate step of alcohol fermentation (*3*−*9*). A minimal reaction mechanism requires a number of steps with potential need for acid−base groups near the active center as seen in Scheme 1. An inspection of the X-ray structures of YPDC from *Saccharomyces uvarum* (*10*) and from *Saccharomyces cerevisiae* (*11*) indicates that aside from the amino group of the 4′-aminopyrimidine ring of ThDP, the reactive side chains of D28, H114, H115, and E477 are near enough to the thiazolium C2H to be potential candidates for converting this carbon acid to the C2-carbanion/ylide to initiate the reaction. We have carried out substitutions at these positions to probe the roles of these amino acids in catalysis. In the first paper in this series, the construction of mutants to yield single amino acid substitutions at these residues and the changes in steady-state kinetic parameters that these substitutions induced were summarized (*12*). Briefly, each residue appears to affect both V_{max} and V_{max}/K_m-type kinetic terms, suggesting that each of the four residues participates in transition state stabilization both at low and at saturating substrate concentration, corresponding to step(s) culminating in the first irreversible step (decarboxylation), and in some steps starting with decarboxylation and culminating in product release, respectively. Interestingly, with some modest exceptions, the shapes of the *V*−pH

† This work was supported by NIH Grant GM-50380, NSF Training Grant BIR 94/13198 in Cellular and Molecular Biodynamics (F.J., PI), and the Rutgers University Busch Biomedical Fund and Roche Diagnostics Corp., Indianapolis, IN. Presented in part at the ASBMB annual meetings in 1999 (*1*) and 2000 (*2*) and at the Enzyme Mechanisms Satellite Meeting of the Australian Biochemical Society, Fraser Island, Queensland, Sep 1999, R. A. Duggleby, Organizing Chair.

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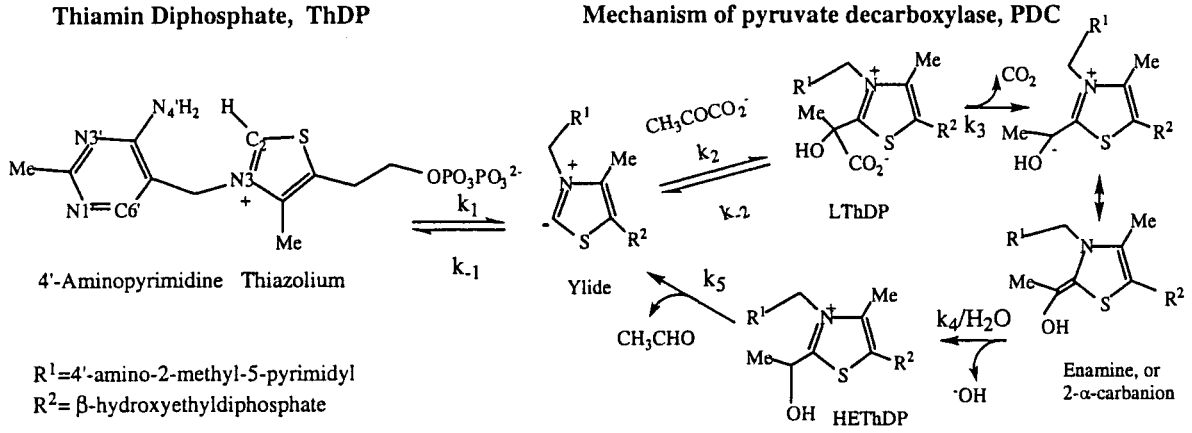
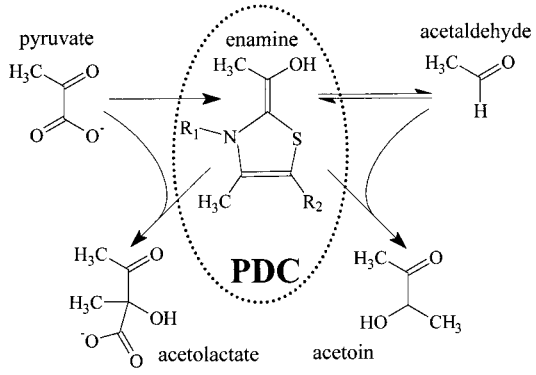
[1] Abbreviations: ThDP, thiamin diphosphate; Aa, acetaldehyde; Ac, acetoin; Al, acetolactate; ADC, acetolactate decarboxylase; ADH, yeast alcohol dehydrogenase; BDH, butanediol dehydrogenase; LDH, lactate dehydrogenase; WT YPDC, wild-type pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae* overexpressed in *E. coli*; E477Q, H114F, H115F, E51D, E51N, E51Q, E51A, D28A, and D28N are variants of this enzyme with the indicated substitutions; ZmYPDC, pyruvate decarboxylase from *Zymomonas mobilis*; ALS, acetolactate synthase (also known as acetohydroxyacid synthase, AHAS); BFD, benzoylformate decarboxylase; HEThDP, C2α-hydroxyethylthiamin diphosphate; LThDP, C2α-lactylthiamin diphosphate; EnThDP or enamine, C2-hydroxyethylidenethiamin diphosphate; BDThDP, C2-(2,3-dihydroxy)-2-*n*-butylthiamin diphosphate, the C2 adduct of ThDP and acetoin; A_{300}, absorbance at 300 nm.

10.1021/bi002856m CCC: $20.00
Published on Web 05/31/2001

Scheme 1: Minimal Mechanism for Pyruvate Decarboxylation by YPDC



Scheme 2: Pyruvate Decarboxylase Catalyzed Reactions



or *V/K*−pH curves were similar for the variants and for the WT enzyme.

In this paper, extensive studies are reported at two positions, D28 and E477, using the 'carboligase' side reactions often observed with all ThDP enzymes. Compared to the usual acetaldehyde formation produced by protonation of the key C2α-carbanion/enamine intermediate, in these reactions the enamine reacts with acetaldehyde, producing acetoin (Ac), or with pyruvate, producing acetolactate (Al, Scheme 2). At the same time, there are ThDP enzymes that deal with these very products: the acetoin dehydrogenase multienzyme complex and acetolactate synthase (ALS; sometimes called acetohydroxyacid synthase, AHAS), respectively, both of which involve the enamine intermediate as well. The carboligase side reaction had already been used some years ago in this laboratory quite effectively to provide mechanistic information about YPDC (*13*). With the availability of the YPDC active center variants, we resorted to study these side reactions once more, seeking more details about the reaction mechanism. As will be seen below, the results indicate that depending on the substitution, the variants become rather effective ALS or acetoin synthases, the side reactions in the WT YPDC often becoming the dominant pathway with the variants. The results provide detailed information on the roles of the two side chains starting with the enamine intermediate and suggest rate-limiting steps. The carboligase products are being formed with significant enantiomeric excess by the YPDC variants. This appears to be the first study in which the rates of formation of both of these side products are being determined with continuous enzymatic assays developed in this laboratory.

EXPERIMENTAL PROCEDURES

Materials. Acetolactate decarboxylase from *Bacillus subtilis* was purchased from Sigma, St. Louis, MO. ALS from plants was a kind gift from Drs. B. K. Singh and Mark Stidham (American Cyanamid, Princeton, NJ). All other reagents were analytical grade or higher.

Enzyme Purification. All YPDC variants were overexpressed in the BL21 strain of *E. coli*. Wild-type YPDC and the E477Q variant have a His_6-tag attached to their C-terminal residues; hence, these were purified on a Talon column (*12*). The D28A and D28N variants were purified as follows. After ultrasonic treatment of the cells in 20 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM ThDP, and 1 mM DTT, the overexpressed enzyme was precipitated with ammonium sulfate (37.5−70%). After dialysis, the enzyme was further purified on an FPLC Mono-Q column (Pharmacia, Sweden) applying a linear gradient of 0−1 M NaCl in 20 mM Bis-Tris (pH 6.8) containing 0.1 mM EDTA, 2 mM $MgCl_2$, and 1 mM DTT. The purity of the enzymes was evaluated by SDS−PAGE and proved to be higher than 95%. [Details are presented in the first paper in this series (*12*).] Butanediol dehydrogenase was purified from dried baker's yeast (Sigma) according to a protocol developed in this laboratory (to be published elsewhere).

YPDC Assays. The rate of acetaldehyde formation was determined by the yeast alcohol dehydrogenase (ADH)/NADH coupled assay (*14*). Detailed description of a continuous assay for determination of acetoin and acetolactate will be published elsewhere. Briefly, acetoin was determined in a coupled reaction with 2,3-butanediol dehydrogenase and monitoring NADH depletion at 340 nm. For determination of acetolactate, a second enzyme, acetolactate decarboxylase, was added to the above assay to convert acetolactate to acetoin. For accurate activity measurements at high pyruvate concentrations, the light path was reduced to 0.5 cm. One unit of activity for acetaldehyde or acetoin or acetolactate formation represents 1 μmol of product produced in 1 min. All the standard activity measurements at a single pH of 6.0 were conducted in 0.1 M MES, pH 6.0, containing 5 mM $MgCl_2$ and 1 mM ThDP at 25 °C unless stated otherwise. Pyruvate-dependent acetoin formation was measured with 100 mM acetaldehyde for the E477Q variant, and with 200 mM acetaldehyde for the D28A and D28N variants. Acetaldehyde-dependent acetoin formation was measured in the

presence of 25 mM pyruvate for the E477Q variant, and with 3.5 mM pyruvate for the D28A and D28N variants.

Only for determination of the pH dependence of the activity, a triple buffer system with constant ionic strength containing 50 mM acetic acid, 50 mM MES, 100 mM Tris adjusted to the pH of interest was utilized, henceforth called 'standard pH-buffer' (*15*). Pyruvate, ThDP, and $MgCl_2$ were added to final concentrations of 20, 1, and 5 mM, respectively. When needed, acetaldehyde was added to the specified final concentration.

Equations Utilized To Fit Experimental Data. Hyperbolic substrate dependencies of the initial velocity were fitted to the Michaelis−Menten equation:

$$v_0 = V_{max}[S]/(K_m + [S]) \quad (1)$$

Cooperative kinetic behavior with inhibition present was described by the Hill equation with substrate inhibition (see eq 1 in the previous paper, ref *12*). In the case of extreme substrate inhibition, where the activity of inhibited enzyme is nonzero, the extended Hill equation was utilized:

$$v_0 = \{V_{max}[S]^n + V_f[S]^{(n+1)}/K_i\}/\{S_{0.5}{}^n + [S]^n(1 + [S]/K_i)\} \quad (2)$$

Circular Dichroism Experiments. To wild-type YPDC or the variant enzymes were added in the standard buffer 5 mM $MgCl_2$, 0.1 mM ThDP, and pyruvate and/or acetaldehyde. The CD spectra were recorded on an AVIV model 202 CD instrument (AVIV Instruments, Inc.). Transient changes due to formation of covalent intermediates were recorded at 4 °C. The steady-state rate of formation of chiral products was measured under the same conditions at 25 °C. The enantiomeric excess of acetoin and acetolactate produced by YPDC variants was estimated from the change in ellipticity at 280 and 300 nm, respectively. All conditions were similar to those used for measuring total acetoin and total acetolactate in the coupled assays. The enantiomeric purity of acetoin was estimated from the values of the molar ellipticity. *These values were related to that of the wild-type YPDC, assuming a 50% enantiomeric excess for acetoin produced by WT YPDC at pH 6.0 (16, 17).*

Rapid-Scan Stopped-Flow Characterization of Covalent Intermediates. Solutions of pyruvate and wild-type or variant YPDCs (the enzymes were preincubated with 50 mM pyruvamide for activation) were mixed on an Applied Photophysics SX.18MV stopped-flow spectrometer in the diode-array mode, and the data were analyzed with the Prokineticist software.

RESULTS

Purification and characterization of variant YPDCs were described in the first paper of this series (*12*).

Acetaldehyde Production by Wild-Type YPDC and Its E477Q and D28A Variants. Typical kinetic results for acetaldehyde release at pH 6.0 (20 mM pyruvate, 20 °C) are as follows. The specific activity of WT YPDC was 40−45 units/mg, that of the E477Q variant was 0.1−0.15 unit/mg, and that of the D28A variant was 0.02−0.03 unit/mg, while the D28N variant had a specific activity of 0.04−0.07 unit/mg. Pyruvate concentration dependencies of the WT YPDC and of the E477Q variant were quite similar and were characterized by $S_{0.5}$ equal to 0.97 mM ($n_H = 1.57$) for WT YPDC and 2.9 mM ($n_H = 1.67$) for the E477Q variant (Figure 1). Slightly higher substrate inhibition was noticed for the E477Q variant at very high pyruvate concentrations ($K_i = 433$ mM for WT and 136 mM for the E477Q variant).

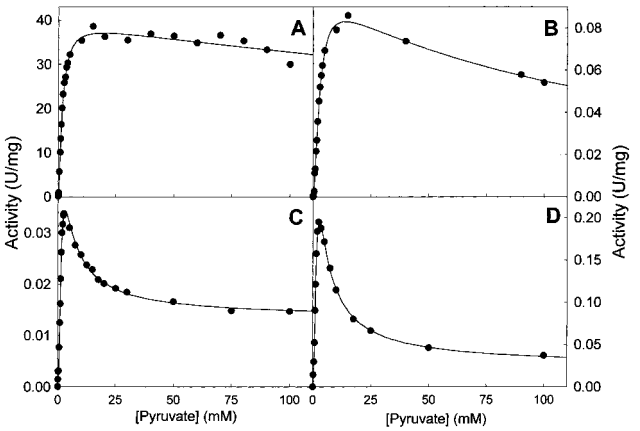


FIGURE 1: Pyruvate-dependent acetaldehyde production by WT YPDC (A) and its E477Q (B), D28A (C), and D28N (D) variants at pH 6.0. Conditions as described under Experimental Procedures. Lines represent the best fit of the experimental data; see parameters under Results.

The pyruvate concentration dependence of the activity of the D28A variant was marked by a sharp rise (Hill coefficient $n_H = 2.1$), followed by a dramatic decrease in the initial rate due to high apparent substrate inhibition ($S_{0.5} = 1.66$ mM and $K_i = 3.04$ mM). The optimal activity (ca. 50% of $V_{max} = 0.065$ unit/mg) was reached for [pyruvate] = 2.5−3 mM. Surprisingly, fully inhibited D28A variant exhibited nonzero activity (0.013 unit/mg), suggesting the coexistence of two pathways for producing acetaldehyde. Pyruvate-dependent Aa formation by the D28N variant was very similar to that produced by the D28A variant, except for a 10-fold higher activity of the former (Figure 1). A sharp increase in activity ($n_H = 1.99$) is followed by a decrease from 0.2 ($V_{max} = 0.69$ unit/mg) to 0.028 unit/mg upon saturation with substrate ($S_{0.5} = 2.48$ mM, $K_i = 1.45$ mM). The activity of 'completely substrate-inhibited' D28N variant (0.028 unit/mg) is double that of the D28A variant, but importantly for the interpretation (see below, also ref *18*, the third paper in this series, as well as ref *19*), for both D28 variants these activities at high substrate concentration were significantly above zero.

Acetoin Production by Wild-Type YPDC and Its E477Q, D28N and D28A Variants. Wild-type YPDC produced small amounts of acetoin compared to acetaldehyde (see Table 1). Addition of exogenous acetaldehyde (to 100 mM) increased the rate of acetoin formation by only about 10%.

The E477Q variant produced acetoin at 18% of the rate of acetaldehyde production. Acetaldehyde added to a concentration of 100 mM to the E477Q variant in the presence of 20 mM pyruvate significantly increased (54-fold) the rate of acetoin formation, leading to an activity 9-fold greater than the rate of acetaldehyde formation (Table 1). Acetaldehyde-dependent acetoin production by the E477Q variant is hyperbolic and is characterized by a $K_m = 5.7$ mM and $V_{max} = 1.02$ units/mg (Figure 2A). The pyruvate concentra-

Table 1: Specific Activities of the Principal and Carboligase Reactions of WT YPDC and Variants at 20 mM Pyruvate (20 °C)

	pH 5.1	pH 6.0	pH 6.9
	WT		
acetaldehyde	22.02	40.73	38.31
acetoin[a]	0.336	0.345	0.330
acetolactate[a]	0.00−0.012	−	−
	E477Q		
acetaldehyde	0.0094	0.079	0.132
acetoin[a]	0.347	0.742	0.705
acetoin[b]	0.009	0.014	0.0367
acetolactate[b]	−	−	−
	D28A		
acetaldehyde	0.0055	0.0089	0.02283
acetoin[a]	0.0222	0.0214	0.024
acetoin[b]	0.0111	0.0096	0.003
acetolactate[a]	0.0726	0.0427	0.0306
acetolactate[b]	0.0805	0.061	0.031
	D28N		
acetaldehyde	ND	0.0712	ND
acetoin[c]	0.260	0.437	0.312
acetolactate[b,d]	0.0557	0.0473	0.0192

[a] Acetoin production is assessed in the presence of 100 mM acetaldehyde. [b] No extra acetaldehyde added. [c] Measured in the presence of 3.5 mM pyruvate and 250 mM acetaldehyde. [d] Measured in the presence of 40 mM pyruvate.

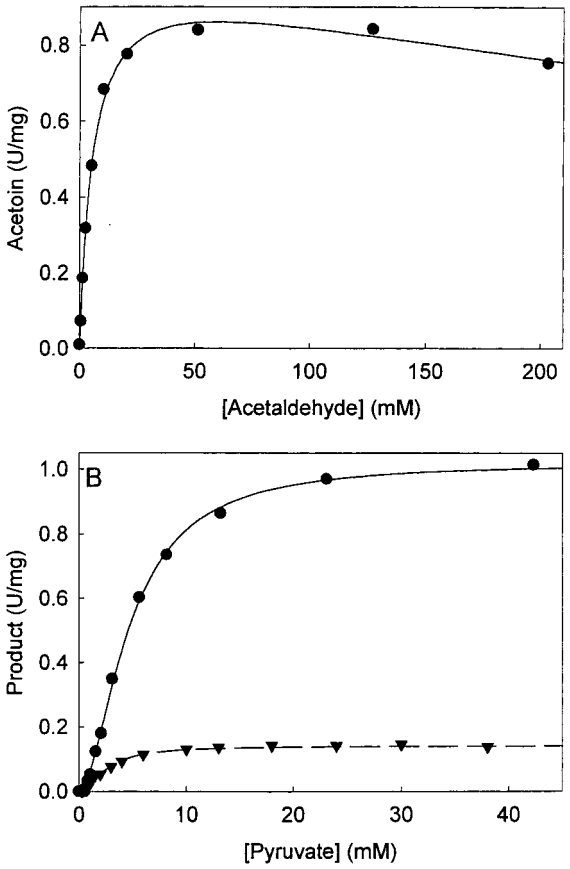


FIGURE 2: Product formation by the E477Q variant YPDC at pH 6.0. Acetaldehyde dependence of acetoin formation is shown in panel A (measured at 25 mM pyruvate), and pyruvate dependence is shown in panel B (closed circles, measured at 100 mM acetaldehyde). Pyruvate-dependent acetaldehyde formation is shown in panel B (triangles) for comparison. Lines represent the best fit of the experimental data; see parameters under Results.

tion dependence of acetoin production was characterized by $S_{0.5} = 4.68$ mM and $n_H = 1.79$ (Figure 2B and Table 2),

Table 2: Kinetic Parameters for Carboligase Reactions of YPDC Variants

Acetaldehyde-Dependent Acetoin Production

	WT	E477Q	D28A	D28N
k_{cat} (s^{-1})	0.345	1.02	0.215	0.737
K_m (mM)	≤5[a]	5.7	50.7	37.9
V/K ($mM^{-1}\,s^{-1}$)	≥0.07	0.18	0.004	0.02

Pyruvate-Dependent Acetoin Production

	WT[b]	E477Q	D28A	D28N
k_{cat} (s^{-1})	(0.345)	1.02	0.230	0.61
V_f (s^{-1})		NA	0.047	ND[c]
$S_{0.5}$ (mM)	(0.97)	4.68	0.96	1.56
n_H	−	1.79	1.87	1.88
K_i (mM)	−	−	12.04	37.8
$V/S_{0.5}$ ($mM^{-1}\,s^{-1}$)	(0.37)	0.47	0.224	0.31 (0.47)[d]

Pyruvate-Dependent Acetolactate Production by D28A

	no acetaldehyde added	200 mM acetaldehyde added
k_{cat} (s^{-1})	0.052 ± 0.0019	0.041 ± 0.00021
$S_{0.5}$ (mM)	3.24 ± 0.31	10.07 ± 0.11
n_H	1.97 ± 0.31	2.10 ± 0.037
$V/S_{0.5}$ ($V/S_{0.5}^n$)[e]	0.0161 (0.0052)[e]	0.00407 (0.00032)[e]

[a] K_m is estimated based on the increment of activity due to added, and expected due to reaction with endogenous acetaldehyde. [b] k_{cat} and $S_{0.5}$ values taken from acetaldehyde-dependent acetoin formation and pyruvate-dependent acetaldehyde formation by WT YPDC. [c] Value could not be determined due to high K_i value. [d] Value in parentheses corresponds to acetaldehyde-dependent V_{max} and pyruvate-dependent $S_{0.5}$. [e] The values of $V/S_{0.5}^n$ are shown in parentheses.

somewhat higher than the values of $S_{0.5} = 2.9$ mM and $n_H = 1.67$ for acetaldehyde production (Table 2).

The D28A variant also produced significant amounts of acetoin (Table 1). The rate of acetoin production was strongly dependent on the presence of acetaldehyde and was equal to 0.23 unit/mg at saturating concentration of acetaldehyde (Figure 3A). Acetaldehyde-dependent acetoin production by the D28A variant is hyperbolic and is characterized by a $K_m = 50.7$ mM. With the D28A variant, the rate of pyruvate-dependent acetoin formation followed the same pattern as the rate of the acetaldehyde-forming reaction. A sharp increase in v_0 ($S_{0.5} = 0.96$ mM, $n_H = 1.87$) is followed by a decrease to a final $V_f = 0.047$ unit/mg due to pyruvate-dependent inhibition ($K_i = 12.0$ mM; see Table 2 and Figure 3B).

The D28N variant produced acetoin with a rate comparable to that for the E477Q variant. V_{max} determined in the presence of 3.5 mM pyruvate is 0.74 unit/mg, that would result in a value of 0.96 unit/mg, were inhibition with pyruvate absent. However, acetoin production was characterized by a $K_m = 37.9$ mM, comparable to that of the D28A variant (Table 2). Pyruvate-dependent acetoin production was also characterized by a Hill coefficient near 1.9, and inhibition by substrate. However, due to its high K_i value, we could not determine the value of V_f. The pyruvate dependence of acetoin production by WT YPDC was not assessed due to fast consumption of pyruvate in the acetaldehyde-forming reaction.

It is important to emphasize that neither the E477Q or D28A variants nor WT YPDC produced measurable acetoin in the absence of pyruvate.

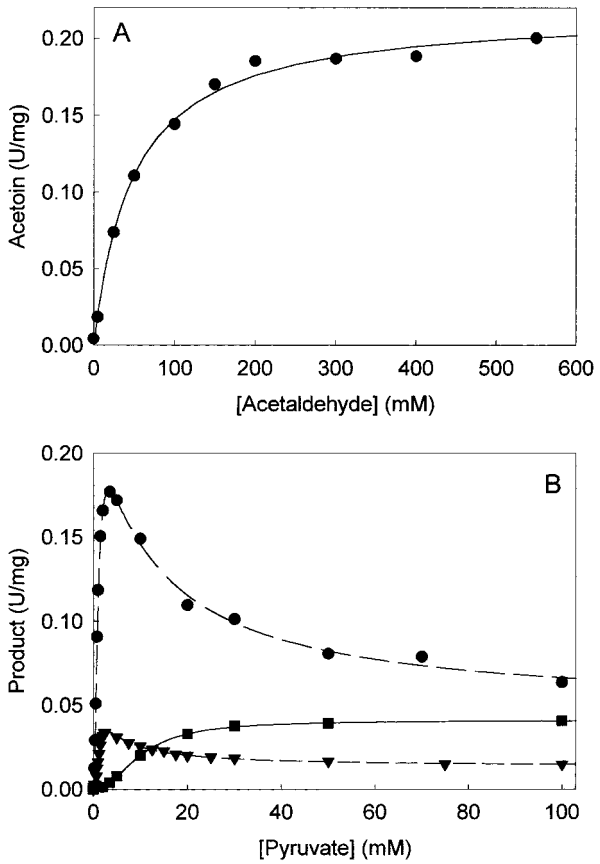


FIGURE 3: Product formation by the D28A variant YPDC at pH 6.0. Acetaldehyde dependence of acetoin formation is shown in panel A (3.5 mM pyruvate present), and pyruvate dependence is shown in panel B (closed circles, 200 mM acetaldehyde present). Pyruvate-dependent acetolactate production was measured in the presence of 200 mM acetaldehyde (closed squares in panel B). Pyruvate-dependent acetaldehyde formation is shown in panel B (triangles) for comparison. Lines represent the best fit of the experimental data; see parameters under Results.

Studies of Acetolactate Production by WT YPDC and the D28A and E477Q Variants. Acetolactate was measured in a coupled reaction with acetolactate decarboxylase (ADC) and/or 2,3-butanediol dehydrogenase (BDH). To calculate the rate of acetolactate formation, the values obtained in the presence of BDH alone were subtracted from the values obtained from the reaction in the presence of both enzymes. This assay provided an accuracy of acetolactate determination at a level of less than 5% of total acetoin/acetolactate concentration. *No detectable acetolactate was produced by the E477Q variant at any pH values studied (pH 5−7).* The WT YPDC may have produced some acetolactate (0.01 unit/mg) at pH 5.1; however, this rate of acetolactate production was at the limit of sensitivity of the assay. On the other hand, the D28A and D28N variants produced mostly acetolactate along with some acetoin.

Pyruvate-dependent acetolactate formation by the D28A variant is characterized by as high a degree of cooperativity ($n_H = 1.97$) as the acetaldehyde-forming activity of the same variant at the same pH of 6.0 (Figure 3B and Table 2). The acetolactate-forming activity at saturating concentration of pyruvate was very similar in the absence (0.052 unit/mg) and presence (0.041 unit/mg) of added acetaldehyde (200 mM). However, addition of acetaldehyde produced significant changes in $S_{0.5}$: it is 3.24 mM in the absence of and 10.1 mM in the presence of 200 mM acetaldehyde.

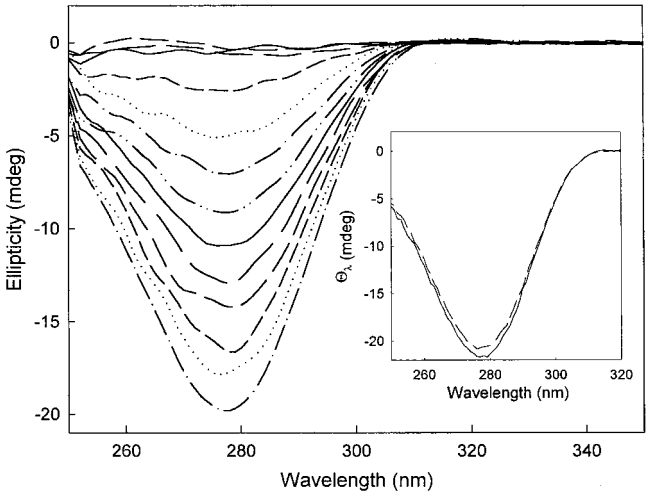


FIGURE 4: CD spectra of acetoin produced by the E477Q variant YPDC. Enzyme (33.5 μg/mL) was dissolved in standard buffer at pH 6.0 with 5 mM $MgCl_2$, 0.1 mM ThDP, and 50 mM acetaldehyde added at 25 °C. Reaction was started with addition of 10 mM pyruvate. Increase in negative ellipticity is due to production of scalemic acetoin. Thermal stability of the product is shown in the inset: solid and dashed lines represent sample before and after boiling, respectively.

The rate of acetolactate formation by the D28N variant under saturating pyruvate concentration was equal to that of the D28A variant. However, due to fast consumption of substrate in the acetaldehyde-forming principal reaction, the substrate dependence of the rate could not be measured.

Circular Dichroism Analysis of the Stereochemical Outcome of Carboligase Reactions. Both the acetoin- and acetolactate-forming carboligase reactions gave rise to optically active compounds. The CD signal of acetoin formed by all variants had a negative sign and a maximum around 278 nm (Figure 4). This signal was insensitive to temperature and retained its magnitude even after boiling for 12 min (see inset to Figure 4). On the other hand, the acetolactate produced by the D28A and D28N variants resulted in a compound with a positive signal and maximum around 303 nm (Figure 5). Boiling of this product resulted in spontaneous decarboxylation, leading to racemic acetoin (inset to Figure 5). The kinetics of carboligase product formation could also be monitored by CD at 280 nm for acetoin and 300 nm for acetolactate. The rate of acetolactate formation by the D28A and D28N variants increased with decreasing pH (Figure 6), opposite in trend to acetaldehyde and acetoin formation rates. The value of the ellipticity produced per unit time per milligram of variant enzyme, combined with the concentration of product formed according to the coupled assay, gave an estimate of the enantiomeric excess produced by each variant (Table 3). The enantiomeric excess of acetoin produced by WT YPDC was 50−58%, and was nearly independent of pH. The enantiomeric excess of acetoin produced by the E477Q variant ranged from 58 to 75%, depending on the pH.

Demonstration of the Presence of Covalent Intermediates Produced on Variant YPDCs. On mixing with pyruvate, the E477Q variant gave rise to a stable absorbance increase in the range of 280−400 nm (Figure 7). Providing that the reaction was run for a prolonged period of time, a steady-

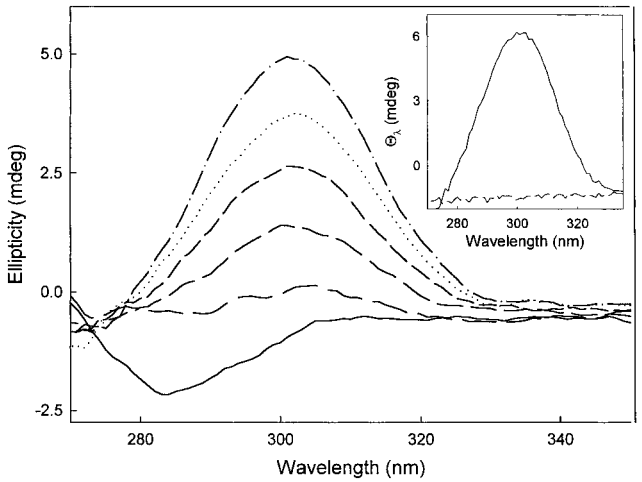


FIGURE 5: CD spectra of acetolactate produced by the D28A variant YPDC. D28A variant (0.13 mg/mL) was incubated in standard buffer at pH 5.1, containing 5 mM $MgCl_2$ and 0.1 mM ThDP at 25 °C. Reaction was started by addition of 20 mM pyruvate. Increase of positive ellipticity at 300 nm monitors acetolactate synthesis. Inset: the characteristic ellipticity (solid line) is lost after boiling for 12 min (dashed line).

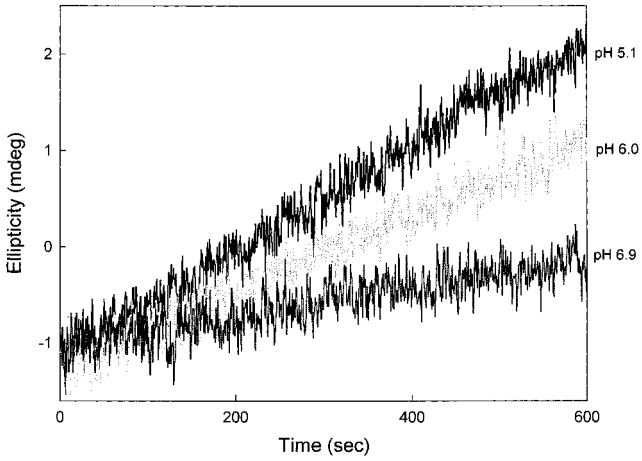


FIGURE 6: Kinetics of stereoselective synthesis of acetolactate by the D28A variant at different pH values. Enzyme (0.133 mg/mL) was dissolved in standard pH buffer at pH 5.1, 6.0, and 6.9, containing 5 mM $MgCl_2$, 0.1 mM ThDP. Reaction was started by addition of 20 mM pyruvate and was monitored via the change in CD signal at 300 nm.

state consumption of pyruvate was also observed (data not shown). Wild-type YPDC under the same conditions did not exhibit the same absorbance increase (Figure 8); however, an initial lag phase was noticeable (inset in Figure 8). At low temperature with the WT YPDC, a small increase was observed in A_{300}; however, its amplitude was at least 5-fold lower than that for the E477Q variant. At the same time, the rate of increase of A_{300} was an order of magnitude slower for the E477Q variant than that for WT YPDC. The amplitude of the A_{300} formed on addition of 1.5 mM pyruvate to the D28A variant was the same as with the WT YPDC; however, the rate of formation was equal to the rate seen with the E477Q variant (data not shown). The absorbance change was only 1.5 times larger with 20 mM pyruvate added.

When YPDC was mixed with pyruvate at 4 °C, the CD spectra underwent fast and significant changes, and these changes were stable at low temperature and were also

Table 3: Stereochemistry of Carboligase Products Formed by YPDC Variants

product		acetoin		acetolactate
variant	pH	molar ellipticity (deg M^{-1} cm^{-1})	enantiomeric excess (%)	molar ellipticity (deg M^{-1} cm^{-1})
WT	5.1	31.09	49.39	
	6.0	31.47	50.00	
	6.9	36.86	58.56	
E477Q	5.1	36.37	57.79	
	6.0	41.39	65.76	
	6.9	47.40	75.31	
D28A	5.1	42.68	67.81	47.13
	6.0	40.19	63.86	47.88
	6.9	40.98	65.11	48.19
D28N	5.1	27.64	43.92	45.89
	6.0	33.69	53.52	46.24
	6.9	41.75	66.34	50.20

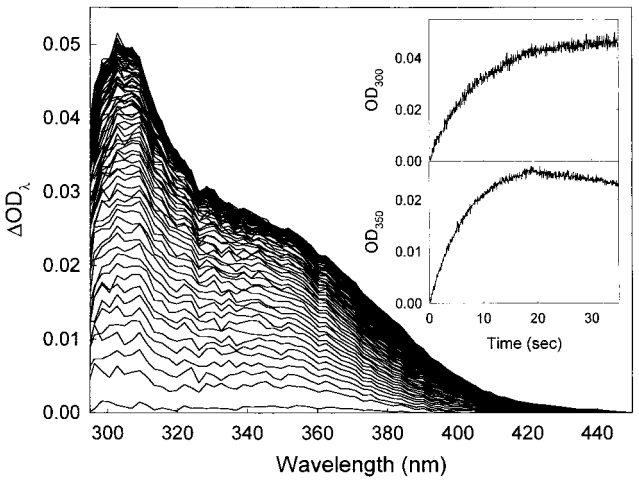


FIGURE 7: Difference spectra of the reaction mixture containing E477Q variant and pyruvate. E477Q variant YPDC (85 μM) preincubated with 50 mM pyruvamide in 100 mM MES, pH 6.0, containing 5 mM $MgCl_2$ and 0.1 mM ThDP, was mixed with an equal volume of 10 mM pyruvate in the same buffer. Spectral changes at 25 °C were recorded using the photodiode-array accessory with the Applied Photophysics SX.18MV stopped-flow spectrometer. Time-dependent changes of absorbance at 300 and 350 nm are shown in the inset.

different for wild type and the E477Q and D28A variants (Figure 9).

pH Dependence of Acetaldehyde, Acetoin, and Acetolactate Production by YPDC Variants. The carboligase activities were examined at pH 5.1, 6.0, and 6.9 for the WT YPDC and the E477Q, D28A, and D28N variants (Table 1). The WT YPDC forms more acetaldehyde at higher pH, attributed to the increase in V_{max} to nearly a plateau value, followed by a decrease above pH 7.0, while the rate of acetoin formation was virtually pH-independent in the pH range examined. An important finding from our study is that the *WT YPDC produced little if any acetolactate.*

The E477Q variant also exhibited a 14-fold increase in acetaldehyde production with an increase in pH from 5.1 to 6.9, and a more modest pH dependence for acetoin formation, with a slight increase from pH 5.1 to 6.0. However, *acetoin production was in excess of acetaldehyde production at all pH values studied, ranging from ca. 30-fold excess at pH 5.1 to 10-fold excess at pH 6.9.*

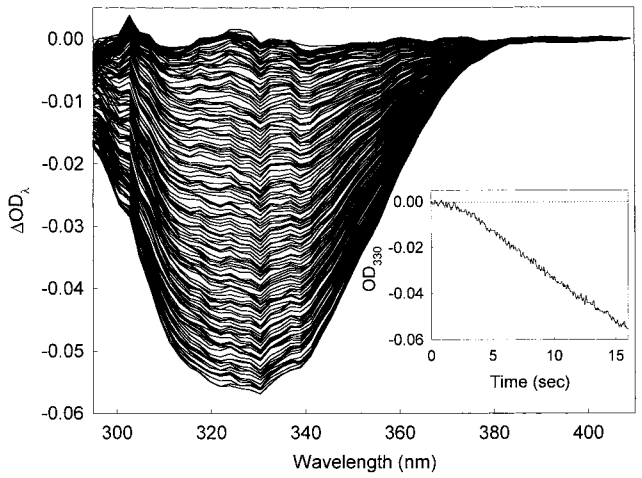


FIGURE 8: UV difference spectra of a reaction mixture containing WT YPDC and pyruvate. Conditions as in Figure 7, except the concentration of the enzyme is 32.2 μM, but also including preincubation with pyruvamide. Time-dependent change at 330 nm is shown in the inset.

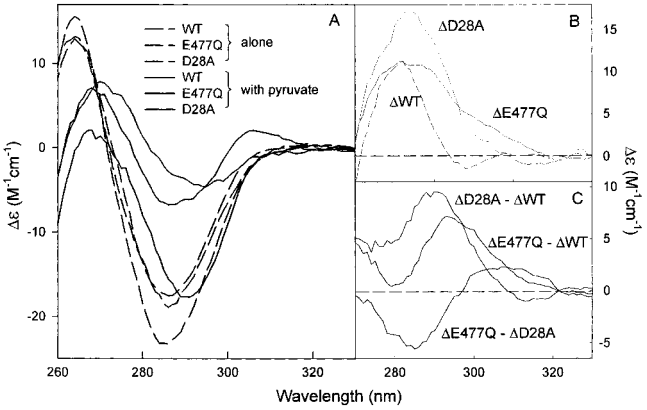


FIGURE 9: CD spectral changes occurring in WT and variant enzymes upon addition of pyruvate (A). WT, E477Q, or D28A at concentrations of 7, 5.7, and 4.9 μM, respectively, were dissolved in standard buffer, pH 6.0, containing 5 mM $MgCl_2$ and 0.1 mM ThDP. After scanning the initial CD spectra, to the enzyme solution was added 10 mM (WT and E477Q) or 1.89 mM (D28A) pyruvate. To ensure minimal interference from carboligase products, the temperature was maintained at 4 °C, and several spectra were collected. Difference spectra (B) and double-difference spectra (C) demonstrate accumulation of distinct intermediates.

The D28A variant also exhibited a 4-fold increase in acetaldehyde formation rate with increasing pH, while acetoin formation did not change from pH 5.1 to 6.9. Only this variant gave clear evidence for acetolactate formation in the entire pH range studied, with ca. 8−10-fold higher rate of acetolactate formation compared to the rate of acetoin formation, and 15-fold higher rate of acetolactate to acetaldehyde formation at pH 5.1, but only a 1.5-fold higher rate at pH 6.9. Unlike the increasing rate of acetaldehyde formation with increasing pH, the rate of acetolactate formation increased with decreasing pH (Figure 6 and Table 1).

DISCUSSION

Wild-type YPDC could produce minute amounts of acetoin and very little, if any, acetolactate (barely within detection limits), along with the major product of pyruvate decarboxylation, acetaldehyde. Observation of the carboligase side products indicates that the enamine intermediate is being partitioned on the enzyme (Scheme 2). The immediate conclusion from the results, confirming the pH dependence and magnitude of k_{cat} of the E477 and D28 variants, is that these substitutions compromise acetaldehyde release. The additional result is that acetoin production is virtually unabated by the substitutions, while substitution at D28 enables the enzyme (unlike the WT YPDC or the E477Q variant) to produce acetolactate.

Acetaldehyde Production by Wild-Type YPDC and Its D28A and E477Q Variants. The variants of yeast YPDC with substitutions at either the E477 or the D28 position exhibited greatly reduced rates of acetaldehyde formation. The k_{cat} values were reduced by ca. 500-fold for the E477Q and D28A variants, and 70-fold for the D28N variant. The $S_{0.5}$ for pyruvate was similar for all variants, in the range of 1.0−2.9 mM. Both substitutions at Asp28 (Ala and Asn) resulted in a larger Hill coefficient than those of the WT YPDC or the E477Q variant.

The significant activity remaining with the D28A and D28N variants allowed us to more closely examine the nature of the high substrate inhibition, so clearly in evidence with several active center variants. The nonzero activity of fully inhibited enzyme species attests to the coexistence of two pathways for producing acetaldehyde (*19*). This topic is addressed in greater detail in the third paper of the series (*18*).

Acetoin Production by Wild-Type YPDC and Its Variants. The WT YPDC and its D28- and E477-substituted variants produced acetoin at different rates, but both pyruvate and acetaldehyde were required components for this reaction. In other words, the *acetoin produced was derived from one molecule of pyruvate and one of acetaldehyde, rather than resulting from the condensation of two pyruvate molecules, followed by decarboxylation of acetolactate*. This is a fundamental finding which prior experimental methods could not settle clearly.

Wild-type YPDC produces only minute amounts of acetoin compared to acetaldehyde. Addition of exogenous acetaldehyde did not significantly alter the amount of acetoin produced, suggesting that the concentration of acetaldehyde produced in the principal reaction was sufficient to keep up with the side reaction. The requirement for participation of acetaldehyde in acetoin synthesis is supported by the CD kinetic experiments (data not shown). In the absence of exogenously added acetaldehyde, the time-course curves for acetoin formation exhibited an initial lag phase. Shortening of the lag phase with increasing enzyme concentration is a result of an increase in the rate of acetaldehyde formation. The lag phase was totally abolished once sufficient exogenous acetaldehyde was added to the reaction mixture.

The high rate of acetaldehyde formation by WT YPDC made it impossible to characterize the dependence of acetoin formation rates on acetaldehyde concentration. The only parameter that was readily available was V_{max}. Therefore, to compare the catalytic efficiency of the WT with that of the variants in this regard, the magnitude of some missing parameters had to be estimated. We based our estimates on three experimentally determined parameters: the rate of acetaldehyde formation and the rates of acetoin formation both in the presence and in the absence of added acetaldehyde. We assumed that the rate of acetaldehyde accumulation is constant. The difference of 10% in the rate of acetoin

production with or without added acetaldehyde, along with the predicted acetaldehyde concentration, gave an estimate for $K_{m(Aa)}$ of approximately 5 mM. However, it must be pointed out that the rate of acetaldehyde formation is likely to decrease, rather than increase (*20*), as a result of acetaldehyde accumulation. The actual value of $K_{m(Aa)}$ is likely to be smaller than the predicted value, and as a result, the $K_{m(Aa)}$ for WT YPDC is smaller than or equal to that of the E477Q variant.

The rate of acetaldehyde-dependent acetoin formation suggests that D28 might participate in the binding of acetaldehyde, since the K_m for acetaldehyde is 8-fold higher for the D28A variant than for the E477Q variant. The value of $V/K_{(Aa)}$ for acetoin formation (Table 2) demonstrates that the E477Q variant makes the most efficient use of acetaldehyde. The D28N variant exhibited properties intermediate between those of the D28A and E477Q variants: it produced large amounts of acetoin at a rate comparable to the E477Q variant; however, it had a lower affinity for acetaldehyde. Its catalytic efficiency for acetoin production was superior to that of D28A, but lower than that of the WT or E477Q variant. The catalytic efficiency of WT is 2.6-fold, and of the D28A variant 36-fold, lower for acetoin formation than that of the E477Q variant. The modest decrease in *V/K* for WT might be a reflection of the uncertainty in $K_{m(Aa)}$ determination. The decreased efficiency exhibited by the D28A variant suggests that it may participate both in binding of acetaldehyde (effect on V_{max}/K_m) and in the release of acetoin (effect on V_{max}). Further substitution of asparagine for alanine at position 28 corrected the effect on V_{max}, but changed *V/K* only marginally for acetoin formation, suggesting that D28 is dissociated (i.e., $D28COO^-$) when it binds acetaldehyde, since the amide group cannot substitute for the carboxylate ion. At the same time, the significant effect on k_{cat} would suggest that the release of acetoin does not require D28 to be dissociated. Since neither the E477Q nor the D28N substitutions decreased the rate of acetoin release, we speculate that deprotonation of the C2α-hydroxyl group of the ThDP-bound acetoin is triggered by the 4′-imino tautomer of ThDP.

Additional information on the role of the active site residues can be obtained from the pyruvate dependence of acetoin formation by different YPDC variants. $V/S_{0.5(pyr)}$ (the closest analogue to *V/K* available) for WT YPDC is expected to be approximately 0.35−0.4 mM^{-1} s^{-1} (based on $S_{0.5}$ of Aa formation and V_{max} for Ac formation), i.e., as high as that for the E477Q and D28N variants, and twice as high as for the D28A variant. This would suggest that if either E477 or D28 participates in the pre-decarboxylation regime of the reaction pathway of the activated enzyme, then there is no change in the ionization state of these side chains, but only the hydrogen bonding properties of D28 are utilized.

Both acetaldehyde- and acetoin-forming activities of the D28A variant exhibit inhibition at high pyruvate concentration, and also exhibit a constant (substrate concentration-independent) rate upon saturation. This suggests that the two alternate electrophilic acceptors (pyruvate and H^+, or pyruvate and acetaldehyde) can be bound to the same enzyme species at the same time. Implications of this finding will be discussed further in the third paper of this series (*18*).

Acetolactate Production by YPDC Variants. It appears likely that WT YPDC can form little, if any, acetolactate at all. Only at pH 5.1 was there any acetolactate observed, essentially at the limits of detection. Both methods of acetoin/acetolactate determination, the older Westerfeld method (*21*) and the one developed in this study, have similar sensitivity for acetolactate determination. Since in both assays acetolactate must first be decarboxylated and its concentration then assessed from the total acetoin concentration, the limit of reliability for acetolactate determination is approximately 3−5% of the total carboligase product.

The inability of the E477Q variant to produce acetolactate suggests either: (a) that access of a second pyruvate molecule to the enamine intermediate is hindered, or (b) that the E477Q-YPDC active site can decarboxylate acetolactate. Arguing against the second explanation is the fact that acetolactate is a β-keto acid whose enzymatic decarboxylation follows entirely different mechanisms, not dependent on ThDP, and, most importantly, that exogenously added acetolactate *is not* decarboxylated by either the E477Q variant or the wild-type YPDC (data not shown). Since acetoin formation by the E477Q variant is greatly diminished in the absence of external acetaldehyde (0.009 unit/mg at pH 5.1), we can be certain that the highest acetolactate formation rate that could go undetected for this variant is 0.0003 unit/mg. On the basis of these arguments, and the apparently lower concentration of post-decarboxylation intermediates present in WT YPDC than in the E477Q variant (see below), we conclude that wild-type YPDC is unlikely to produce acetolactate.

Pyruvate-dependent acetolactate production by the D28A variant demonstrates that acetaldehyde and pyruvate as electrophilic acceptor molecules of the enamine likely compete for the same binding site. We conclude this on the basis of $S_{0.5}$ for pyruvate being the only parameter that is affected by the presence of Aa, whereas V_{max} and the Hill coefficient were little changed.

Direct Observation of Covalent Intermediates Bound to YPDC Variants. Absorption spectral changes observed on addition of pyruvate to the WT YPDC and the D28A variant indicate that there is no significant enamine accumulated on these enzymes (expected maximum at 295−310 nm; see ref *22*). The amplitude of the increase in A_{300} for WT YPDC and the D28A variant was at least 5-fold smaller than for the E477Q variant. For simplicity, let us assume that the enamine intermediate is formed with a rate constant k_1 (decarboxylation), and is consumed in the subsequent reaction with a rate constant k_2 (enamine protonation to give HEThDP), leading to an amplitude for $A_{300} = \epsilon_\lambda E_t/(1 - k_2/k_1)$. The maximum value of the amplitude equals $\epsilon_\lambda E_t$ for $k_2 = 0$. If we assume that the E477Q variant behaves in this manner, with a much smaller enamine depletion rate than the rate of its formation, then WT YPDC and the D28A variant are characterized by a rate of enamine formation at least 6 times smaller than its depletion. At the same time, the magnitude of A_{300} for the D28A variant can be explained, if this variant never achieves maximal velocity, and the catalytic rate even at the optimum pyruvate concentration of 2.5−3.0 mM is only half of the potential V_{max} of the enzyme. Thus, it is possible that in the absence of inhibition with substrate, the concentration of enamine could be larger. Addition of higher pyruvate concentration did increase the A_{300}, supporting this idea.

The E477Q variant was the only enzyme form studied that gave clear evidence of a significant absorbance increase in the broad range of 280−400 nm, most likely due to the accumulation of covalent intermediate(s) (Figure 7). Subsequent steady-state consumption of pyruvate is much slower than that catalyzed by WT YPDC. There is evidence from data such as those in Figure 7 to signal the presence of at least one long-lived transient on the E477Q variant. While we hesitate to assign structure to this transient (but see CD results in the following paragraph), there are only two highly conjugated structures that may account for the observations: the enamine shown in Scheme 1 (*22*) and/or some modified form of the coenzyme (not shown), such as the imino tautomer.

CD spectral changes upon reaction with pyruvate were observed even with WT YPDC and take place mostly below 290 nm; these changes are likely the result of a conformational change that occurs on activation with substrate. The two variant enzymes exhibited *transient* CD changes different from those of WT and from each other (Figure 9A). To differentiate the CD shift due to a conformational change (similar to WT) from changes due to the presence of intermediates, we generated a series of CD difference spectra. In Figure 9B are shown differences for the three enzyme forms in the absence and presence of saturating pyruvate (ΔWT, ΔD28A, ΔE477Q). In Figure 9C, we generated double-difference spectra, in which we looked for a difference between WT and variant (ΔD28A − ΔWT, ΔE477Q − ΔWT) and, finally, between the two variants themselves (ΔE477Q − ΔD28A). As is clearly seen in Figure 9, each variant gave rise to its own characteristic CD shift, likely due to accumulation of different intermediate species. Without a doubt, these intermediate species are chiral (chirality either being induced by the environment or simply due to the presence of a chiral center). Again, attention is called to the spectrum ΔE477Q − ΔD28A, presumably representing an intermediate present in greater concentration on the E477Q variant with Θ_{max} near 310 nm. Because of the model studies on enamine structures, we believe that the most likely assignment, consistent with the absorption spectroscopic evidence above, is to the enamine. This has very profound mechanistic implications, but for the moment, it clearly suggests that substitution at E477 seriously interferes with enamine protonation.

Deductions Concerning the State of Ionization of Active Center Residues on YPDC. The catalytic efficiency of the E477Q variant for acetoin production was largely unchanged, whereas acetaldehyde production was greatly suppressed compared to WT YPDC. This observation suggests that in the wild-type YPDC, the E477 residue is likely to be important in the release of acetaldehyde, perhaps protonating the enamine intermediate (Scheme 1).

Replacement of D28 with alanine enabled the enzyme to synthesize acetolactate with an efficiency somewhat lower than that for acetoin production by either WT YPDC or the E477Q variant. Within experimental error, neither WT YPDC nor E477Q could produce acetolactate. We realized that either the diminished size or the altered charge of the D28A substitution could result in easier access of the second pyruvate to the enamine. The two alternatives could be sorted out since the D28N variant could also synthesize acetolactate with efficacy comparable to that of the D28A variant, suggesting that in the WT enzyme it is the negative charge, rather than the size of the D28 side chain, that prevents it from producing acetolactate. Therefore, we propose that at the enamine stage of the reaction, awaiting the carboligase side reaction, D28 exists in the dissociated ionization state, i.e., as $D28COO^-$. This conclusion is consistent with the above demonstration that the $D28COO^-$ form acts as the binding site for acetaldehyde substrate (see discussion on acetoin production etc. in preceding paragraphs).

A decrease in pH diminished the rate of acetaldehyde production, at the same time increasing the rate of acetolactate production by the D28A variant, with the ratio of rates for (Ac + Al)/Aa reaching 17 at pH 5.1. This would imply that access of the second pyruvate (in contrast to the first pyruvate resulting in enamine formation) is greatly facilitated. At the same time, acetoin production in the presence of high acetaldehyde concentration was largely unchanged with a decrease in pH from 6.9 to 5.1, since acetaldehyde is uncharged. The increasing rate of acetolactate formation with decreasing pH is probably due to the protonation of a nearby group at the active site, probably H114 or H115, or of the pyruvate itself.

Apparently, in WT YPDC the state of ionization is D28COOH through decarboxylation (for conversion of the first pyruvate), and is switched to $D28COO^-$ post-decarboxylation to help avoid build-up of acetolactate by repelling the second pyruvate, and acting as a "gate-keeper" of the active site to protect it from an undesirable side reaction. In the ground state, before substrate enters the YPDC active site (*9*), there is a Cl^- ion hydrogen-bonded to H115, which in turn is also hydrogen-bonded to the main chain carbonyl oxygen of W412. This requires that H115 bears a proton at each imidazole nitrogen; i.e., it is in the hisitidinium ionization state. That same Cl^- ion is <4 Å from the D28 carboxyl oxygens, suggesting that, at least prior to the reaction, D28 is undissociated. We suggest that the weight of the evidence is that once decarboxylation is complete, D28 is dissociated by transferring a proton, perhaps to E477, via an intervening water molecule. It is possible that a negatively charged D28 interacts with protonated H115 in WT YPDC, forming an ion pair at the enamine stage, and this positive charge at H115 is exposed in the D28A and D28N variants, thereby attracting the second pyruvate to form acetolactate. So far, we have not identified a mechanism for releasing a hydroxide ion to solution (as dictated by the stoichiometry of the overall reaction); one possible mechanism, conversion of CO_2 to HCO_3^-, has been ruled out for all enzyme forms studied here (*12*).

Our working hypothesis for the state of ionization of the active center of YPDC pre- and post-decarboxylation is the following:

pre-decarboxylation:

$$E477COO^-...D28COOH...H^+His115$$

post-decarboxylation:

$$E477COOH...D28COO^-...H^+His115$$

We have no experimental data relevant to the state of ionization of H114.

It is an important observation in the first paper in this series (*12*) that k_{cat}−pH and $k_{cat}/S_{0.5}$−pH plots have similar shapes

in the wild-type and D28A, H114F, H115F, and E477Q variant forms of YPDC. While several modest shifts were noted, the behavior of the D28A variant in exhibiting an alkaline shift of the acid limb of the k_{cat}−pH plot (monitoring kinetic barriers at saturating substrate levels), and an acid shift of the acid limb of the $k_{cat}/S_{0.5}^{n}$−pH plot (monitoring kinetic barriers at low substrate concentrations), is an exception, also suggesting that D28 exists in different ionization states pre- and post-decarboxylation. Interestingly, residue S26 of benzoylformate decarboxylase [BFD, an enzyme with very similar function to that of YPDC in which the substrate methyl group is replaced by a phenyl group (*23*)] is located at the position corresponding to D28 in YPDC. Almost certainly S26 in BFD exists in its alcohol, rather than alkoxide, ionization state throughout the catalytic cycle. This would also be consistent with D28 having a role in which it is undissociated pre-decarboxylation. It would also mean that dissociation of D28 is not important for the chemistry of the reaction, and the sole reason for this dissociation may be to protect the active site from inhibitory substrate, a function not needed by BFD, given the larger size of the BFD substrate.

Stereochemical Conclusions. The CD experiments have turned out to be of great benefit both at substrate and at enzyme concentration levels. These experiments gave clear evidence of the formation of nonracemic acetoin and acetolactate. They were also useful in kinetic studies. While the formation of nonracemic acetoin product has been in the literature for many years (ref *11* and references cited therein), this appears to be the first clear-cut evidence for formation of acetolactate with the D28A and D28N variants with significant enantiomeric excess.

As was previously reported, the NAD-dependent 2,3-butanediol dehydrogenase reduces the (*R*)- and *(S)*-acetoin equally efficiently, such that reduction of a racemic mixture of acetoin leads to a 1:1 mixture of (*R,R*)-, and *meso*-2,3-butanediols (*24*). This ensures that in the coupled reaction used, the total concentration of acetoin or acetolactate was being measured. Having determined the total concentration of acetoin produced, and with data on the enantiomeric excess produced by WT YPDC as a reference, the CD results allowed us to conclude that the E477Q and D28A variants also produced (*R*)-acetoin (presumably by attack of the enamine on the *si*-side of acetaldehyde) with enantiomeric excess comparable to that produced by the WT YPDC.

Had residues E477 and D28 any role in determining either the orientation of attack or the magnitude of the enantiomeric excess of acetoin produced, we may have expected the results to be different. As Figure 10 illustrates, the results were very similar with the variants as with the WT YPDC and varied little with pH. We conclude that neither D28 nor E477 directly controls the stereoselectivity of carboligation by orientation of the acetaldehyde (either hydrogen bonding to the carbonyl oxygen or, especially, proton transfer to the incipient alkoxide). This may appear contradictory to the previous suggestion that D28 assists acetaldehyde binding; however, the K_m is a reflection of the strength of the Michaelis complexes (or, rather, of the relative distribution of enzyme between the free and substrate-bound forms). The enantiomeric excess, on the other hand, depends on the docking position, and the direction of attack by the acetaldehyde. Thus, the results imply that acetaldehyde binding

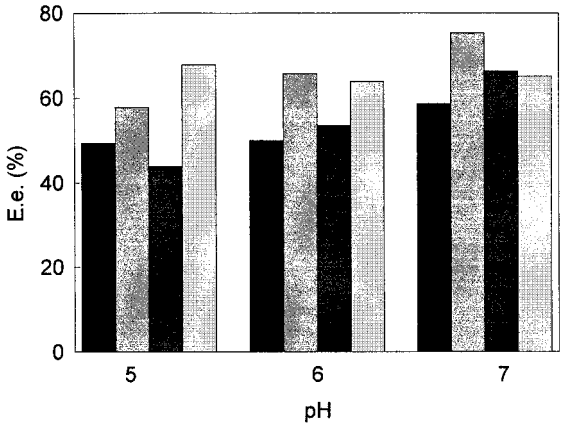


FIGURE 10: Stereoselective production of acetoin by WT YPDC and the variants. Enantiomeric excess of (*R*)-acetoin was calculated based on the molar ellipticity of the acetoin produced by different variants with acetoin produced by WT YPDC at pH 6.0 as a reference for 50% enantiomeric excess. In each group from left to right, the enzymes are WT, E477Q, D28N, and D28A.

at the active center does not ensure either being in the proper orientation or being poised for attack; rather, it is likely that an additional reorientation step in the active center is required prior to bond formation.

Comparison of the CD spectra of acetolactate produced by plant acetolactate synthase and by the two D28 YPDC variants (Figure 11) lead us to conclude that all three enzymes predominantly produce the same (*S*)-acetolactate enantiomer (see the inset to Figure 11, and ref *25*), as we predicted earlier (*2*). This means that attack on the pyruvate molecule must take place from the *si*-side. With the carbonyl oxygen of either acetaldehyde or the second pyruvate molecule as the locus of interaction with the active site, this docking mode ensures that the methyl and carboxyl group of the second pyruvate take the place of the hydrogen and methyl group of acetaldehyde, respectively. Therefore, we conclude that *it is the size of the group that determines the orientation of the electrophilic acceptor molecule in the carboligation reaction*. It is also possible that, coincidentally, the larger negatively charged carboxylate group in pyruvate imparts an additional driving force for the observed orientation.

The fact that both substitutions at D28 led to formation of acetolactate of virtually the same enantiomeric excess (Figure 11) suggests that the size of the D28 side chain is much less important than the neutral charge in allowing the second pyruvate molecule to enter the active center. Again, the enantiomeric excess is likely controlled by factors other than D28, but the charge at D28 is absolutely required. It is also noteworthy that the rate of acetolactate formation is virtually the same for the D28A and D28N variants, but it is approximately an order of magnitude lower than the rate of acetoin formation for the same two variants, consistent with aldehydes being better electrophiles than ketones, in general.

We also note that the enzyme acetolactate synthase produces the same configuration of acetolactate as YPDC (*25*), affirming that the preferred approach of pyruvate to the enamine must be conserved on the two enzymes, even though there is only 20% sequence identity overall.

Surprisingly, the activity of the D28 variants for acetolactate formation was higher than that of ALS from some sources (*26*).

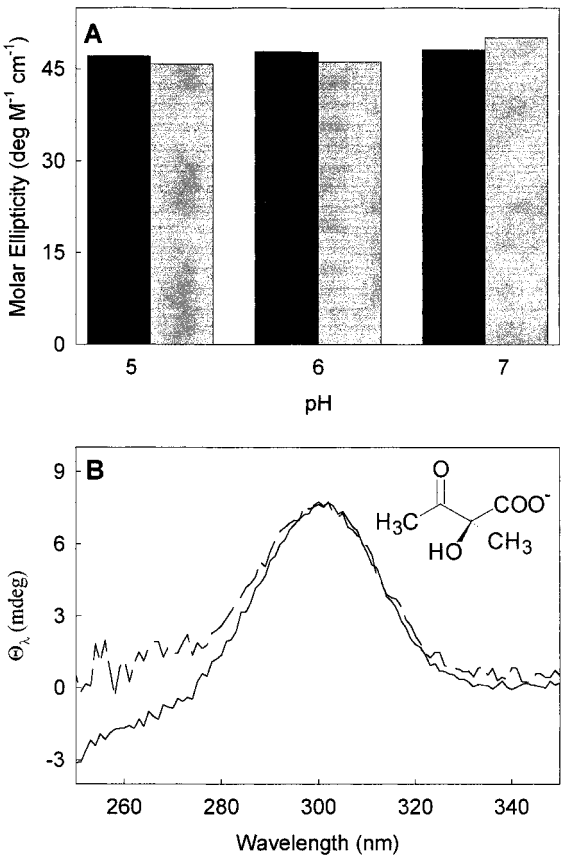
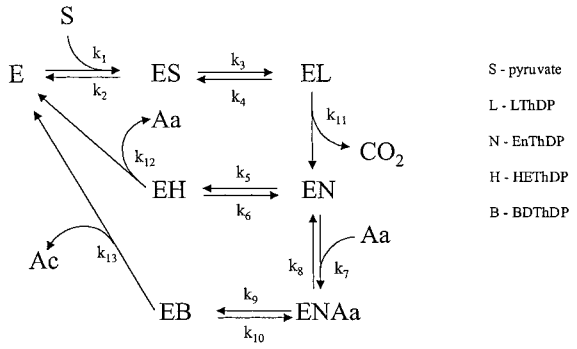


FIGURE 11: Stereoselectivity of acetolactate production by the D28A and D28N variants. Molar ellipticity of acetolactate (A) produced by D28A (dark bars) and D28N (light bars) was measured in standard pH buffer containing 0.1 mM ThDP, 5 mM $MgCl_2$, and 20 mM pyruvate. The spectrum of acetolactate (B) produced by D28A (solid line) displayed the same ellipticity as acetolactate produced by plant acetolactate synthase (dashed line). Conditions for the D28A variant were as in Figure 5. Acetolactate synthase (0.79 mg/mL) was incubated with 40 mM Tris-HCl (pH 7.0), 8 mM $MgCl_2$, 0.8 mM ThDP, 5 μM FAD, and 200 mM pyruvate overnight at 25 °C. The sample was diluted 13-fold in water for recording the CD spectrum. The structure of (*S*)-acetolactate is shown in the inset to panel B.

Rate-Limiting Step of the D28A, D28N, and E477Q YPDC Variants. Understanding why the E477Q variant produces acetoin 10 times faster than it forms acetaldehyde, and why it produces acetoin 2.5 times faster than does WT YPDC, could elucidate important aspects of the mechanism. One might argue that this is just a reflection of the competition of the two electrophiles for the same enamine species. Scheme 3 presents the mechanism usually written for the carboligase reactions, with acetaldehyde (Aa; the step with rate constant k_{12}) and acetoin (Ac; the step with rate constant k_{13}) products. In this branched reaction, the enamine either can undergo protonation (followed by acetaldehyde release) or can attack acetaldehyde to form 2-(2,3-butanediol)-ThDP (BDThDP), therefore releasing acetoin. E and S represent activated enzyme and pyruvate. Acetolactate formation can also be described by this scheme if pyruvate (S) replaces acetaldehyde in the binding step denoted by the rate constant k_7.

The reactions forming acetoin and acetolactate compete with the enamine protonation−deprotonation reaction (k_5 and k_6) and acetaldehyde release from HEThDP (k_{12}). However, all the post-decarboxylation reactions to form acetaldehyde

Scheme 3



are first order, and therefore they will not be able to compete with a second-order reaction, k_7[Aa] (or k_7[S] for acetolactate formation), when the concentration of second substrate (acetaldehyde or pyruvate) is saturating. Therefore, even intuitively, the values of k_5, k_6, and k_{12} will not bear any significance for the V_{max} of the carboligase reaction measured at saturating concentration of second substrate, and the rate of acetoin formation is expected to be independent of the rate of acetaldehyde formation.

To demonstrate the point, we compare the rate equations describing V_{max} for acetoin and acetaldehyde formation. Under conditions of saturating pyruvate and acetaldehyde concentrations, the rate of acetoin formation is

$$V_{max}^{(Ac)} = 1/(k_4/k_3k_{11} + 1/k_3 + 1/k_{11} + 1/k_9 + k_{10}/k_9k_{13} + 1/k_{13}) \quad (3)$$

where k_3 and k_4 are the rate constants of LThDP formation and its reversal to enzyme−substrate complex, respectively, k_{11} is the rate constant for decarboxylation, k_9 and k_{10} are the rate constants for 2-(2,3-butanediol)-ThDP formation and its reversal to enamine−enzyme−acetaldehyde complex, and, finally, k_{13} is the rate constant for acetoin release.

The rate of acetaldehyde formation (in the absence of external acetaldehyde added) is given by the equation:

$$V_{max}^{(Aa)} = 1/(k_4/k_3k_{11} + 1/k_3 + 1/k_{11} + 1/k_5 + k_6/k_5k_{12} + 1/k_{12}) \quad (4)$$

The E477Q variant can produce acetoin 10 times faster than acetaldehyde. From a comparison of eqs 3 and 4, one can deduce that in order for $V_{max}^{(Ac)} \gg V_{max}^{(Aa)}$ there must be a unique relationship of rate constants as follows:

$$1/k_5 + 1/k_{12} + k_6/k_5k_{12} \gg 1/k_9 + 1/k_{13} + k_{10}/k_9k_{13}$$

This comparison implies that the rate constant for each forward post-decarboxylation step on the acetaldehyde-forming pathway is smaller than the corresponding rate constant on the acetoin-forming branch, but is larger on the reverse post-decarboxylation steps than on the corresponding acetoin-forming branch. The two branches share the same pathway through decarboxylation. If the rate of decarboxylation, or any of the steps preceding it, is rate-limiting in acetaldehyde formation, one would not expect the rate of acetoin production to be higher than the rate of acetaldehyde formation. In other words, the rate-limiting step for the acetaldehyde-forming branch must be in the post-decarboxylation phase. Indeed, in order for the pre-dacarboxylation or

decarboxylation phase to be rate-limiting, the following must hold: $(k_4/k_3k_{11} + 1/k_3 + 1/k_{11}) > (1/k_5 + k_6/k_5k_{12} + 1/k_{12})$. Then the following inequality also must be obeyed: $(k_4/k_3k_{11} + 1/k_3 + 1/k_{11}) \gg (1/k_9 + 1/k_{13} + k_{10}/k_9k_{13})$, suggesting that for pre- or decarboxylation to be rate-limiting for acetoin production the maximal rates would be expected to be the same in both branches, which is not the case.

The E477Q and D28A variants both exhibited a higher acetoin-forming rate than acetaldehyde-forming rate (Tables 1 and 2). Therefore, both variants have higher rates pre-decarboxylation than post-decarboxylation in the acetaldehyde-forming branch (i.e., post-decarboxylation is rate-limiting in the overall reaction). And *both the E477Q and D28A variants are expected to have higher steady-state concentrations of post-decarboxylation intermediates (enamine, HEThDP, or enzyme−product complex) than pre-decarboxylation ones (enzyme−substrate Michaelis complex or LThDP)*.

On addition of pyruvate, both the E477Q and D28A variants showed the presence of intermediates with CD features distinct from that of WT YPDC, and also distinct from each other. At the same time, the E477Q variant was the only one to give clear evidence of a stable intermediate with an absorbance maximum around 305 nm. On the basis of model systems, we are tempted to assign the absorbance to the enamine intermediate. With this tentative assignment, and on the basis of the amplitude of the A_{300}, the D28A variants possessed as little as one-fifth of the concentration of enamine present on the E477Q variant, suggesting the D28A variant will accumulate 20% of enamine and 80% of HEThDP, since binding of acetaldehyde is greatly impaired for this variant and we can hardly expect it to accumulate enzyme−product (acetaldehyde) complex.

The A_{300} for wild-type YPDC was comparable to that with the D28A variant; therefore, the wild-type YPDC might also be expected to have as much as 20% of enamine present in steady state. However, this number is not firm, partly due to a fast decrease in the absorbance that might be simply due to a steady-state consumption of pyruvate (maximum absorbance at 320 nm) or, perhaps, to a transient consumption of enamine through protonation and acetaldehyde release before WT YPDC reaches steady-state concentrations of the covalent intermediates. The observation of CD transients with different spectral characteristics on mixing pyruvate with the D28A variant or WT YPDC supports the latter explanation and might imply that WT YPDC accumulates pre-decarboxylation intermediates, rather than post-decarboxylation intermediates. *The data also suggest that, while both D28 and E477 may participate in enamine protonation, D28 may also participate in acetaldehyde release*.

Another observation that deserves special consideration is the rate of acetoin formation by WT and the E477Q variant YPDC. As can be seen from eq 3, the rate of acetoin formation does not include the rates of HEThDP protonation/deprotonation and acetaldehyde release. That is, in the WT YPDC with 'normal' acetaldehyde release, and with the E477Q variant where acetaldehyde release is impaired, the rates of acetoin release are expected to be equal if all other reactions are unperturbed. However, we found that the rate of acetoin production by the E477Q variant is 2.5-fold higher than that observed with the WT YPDC. This might be a result of a decrease in k_4 or k_{10}, or an increase in k_3, k_9, k_{11}, or k_{13}, enabling the E477Q variant to produce acetoin at a higher rate. But, any plausible explanation would invoke increased throughput of the acetoin-forming branch, difficult to accept for a variant with greatly reduced overall activity. We propose instead, that it is not the rate of any particular step that determines the increased rate of acetoin formation by the E477Q variant, but rather a change in the YPDC mechanism [for more details, please see the third paper in the series (*18*)], leading to the accumulation of post-decarboxylation intermediates.

Preliminary results show that both the H114F and H115F variants could also produce acetoin, but not acetolactate, with the overall activity much less than displayed by the D28N and E477Q variants. The results show that while both the H114 and H115 side chains are beneficial to acetoin formation, neither residue is essential. At the same time, both H114F and H115F variants exhibited smaller rates of acetaldehyde formation than acetoin formation, implying that for those variants the post-decarboxylation part of the principal reaction is rate-limiting.

In contrast, among the E51Q, E51D, E51N, and E51A variants (produced in our laboratory; see ref *27*), only the E51A variant could synthesize some acetoin with an order of magnitude lower activity than for acetaldehyde formation. This result shows that residue E51 is indispensable for acetoin formation, almost certainly due to the importance of this side chain in maintaining the amino−imino tautomerization capability of the coenzyme.

Clearly, substitution of the active site D28, E477, H114, and H115 residues is more damaging for acetaldehyde formation than for acetoin formation. The finding that all four variants could still produce acetoin clearly attests to the fact that none of the four is absolutely required for docking acetaldehyde. With the E51 variants, the behavior is different, since the substitutions are equally damaging for both activities, once again supporting our suggestion concerning the crucial role of the *N*4′-amino group for acetoin formation and release. It appears likely that for the post-decarboxylation steps of acetaldehyde formation, unaltered D28, E477, H114, and H115 residues are equally important, whereas for acetoin formation the functional ability of the aminopyrimidine ring is more important. *We therefore conclude that the N4′-amino group is the most likely essential candidate for docking acetaldehyde and also for controlling the stereochemical outcome of the reaction.*

Also consistent with this argument is the sequence alignment of ALS from different sources with the sequences of POX, YPDC, and ZmPDC, showing that only pyruvate decarboxylases possess D28 and E477. These two positions are preceded by highly homologous regions in pyruvate decarboxylases and acetolactate synthases. Both YPDC and ZmPDC have Glu in the same position (E477 in YPDC and E473 in ZmPDC), but ALS from all sources have Gln (*28*), while POX from *Lactobacillus plantarum* has Ala in the corresponding positions. The N-terminal residues 4−63 of YPDC have 35% identity and 55% strong homology with the region of residues 92−151 of yeast ALS. However, residue D28 of YPDC is replaced by an Ala residue in the ALS. This once again affirms that in pyruvate decarboxylases the D28 and E477 are very likely involved in product release, steps that are not needed in either POX or ALS.

Functional vs Structural Sites for Substrate on YPDC. Several decades ago, Juni (*20*) proposed the existence of separate "enzymatic sites" being responsible for decarboxylation of pyruvate and the release of acetaldehyde or carboligase reaction on YPDC. This conclusion was deduced on the basis of acetolactate formation and the inability to release acetaldehyde in ThDP model reactions in the absence of enzyme, in contrast to acetaldehyde formation catalyzed by YPDC. Different sensitivity of the principal reaction and the carboligase side reaction to limited proteolysis of YPDC was also attributed to the presence of two separate sites (*29*, *30*). On the basis of results here reported, the correct explanation is likely to be different. Namely, substitution of several amino acids in the active site led to a 500−1000-fold decrease in acetaldehyde formation, yet either unchanged or even enhanced ability for carboligase-type reactions. Taking into account the additional data concerning the accumulation of covalent intermediates on the variant enzymes, we suggest that the results of Juni and co-workers can be explained by alteration in the active site charge distribution and/or hydrogen bonding patterns during the pre- and post-decarboxylation phases of the reaction. More than likely, the first part of the reaction is more heavily dependent for catalysis on ThDP itself, whereas steps after decarboxylation require facultative participation of active site amino acids. The same explanation would suffice if YPDC altered by proteolysis carried out steps through enamine formation and carboligase reactions, but led to greatly compromised release of acetaldehyde.

CONCLUSIONS

The assays developed here enabled us to study the rates of both carboligase side reactions and to draw mechanistic details about the YPDC active center residues, not readily available from the steady-state kinetic data reported in the first paper of this series (*12*). Most significantly, it was convincingly demonstrated that substitution at D28 only (but not at E477, or with the wild-type YPDC) enables formation of acetolactate, the D28A and D28N variants thus becoming efficient acetolactate synthases. Conditions were also identified under which the E477Q variant was converted to an acetoin synthase. The further demonstration that both acetoin and acetolactate are being produced with high enantiomeric excess by these relatively simple applications of protein engineering makes these substitutions particularly powerful for future work. In addition, the results provide strong mechanistic arguments for the importance of D28 and E477, as well as of H114 and H115, in post-decarboxylation events, and more modest impact on pre-decarboxylation events.

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BI002856M